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Photograph of Dr. Benjamin Franklin, F.R.S., from the Painting in the possession of the Royal Society. Mr. Hyatt.

"On the Demonstration of the Presence of Iron in Chromatin by Micro-Chemical Methods." By A. B. MACALLUM, M.B., Ph.D., Lecturer in Physiology in the University of Toronto. Communicated by Dr. H. NEWELL MARTIN, F.R.S. Received April 23,—Read April 30, 1891. Revised August, 1891.

The investigation, some of the results of which are to be given in the present paper, was stimulated by the conclusions of studies which I carried on during the last five years, and by the observations of Bunge* and Zaleski† on the occurrence of iron-holding proteids in the food and in the liver. The conclusions which I drew from my studies were :—

(1.) That in Amphibia the hæmoglobin is derived from the very abundant chromatin of the hæmatoblasts.‡

(2.) That in the maturing ova of Amphibia the abundant chromatin of the nucleus diffuses out from the latter, and, as the diffusion commences and progresses, the yolk spherules appear and increase in size. In other words, chromatin constitutes a large part of each yolk spherule.

(3.) That there is a transference of chromatin from the maternal tissues to the fœtal villi in the placenta of the Cat, and that this chromatin is carried from the central portions of the villi by the amœboid cells of the latter onwards towards the fœtus.

From the first conclusion it follows that the chromatin of hæmatoblasts contains iron. A consideration of the remaining conclusions points, however, to a wider distribution of iron. Bunge found in the yolk of the hen's egg an iron-holding nuclein, which, from its

* "Ueber die Assimilation des Eisens," 'Zeit. f. Physiol. Chemie,' vol. 9, p. 49.

† "Studien über die Leber.—1. Eisengehalt der Leber," 'Zeit. f. Physiol. Chemie,' vol. 10, p. 453.

‡ The observations on this subject are described in a paper which is to appear in the forthcoming number of the 'Transactions of the Canadian Institute.'

supposed connexion with hæmatopoiesis in the developing chick, he named hæmatogen. In the amphibian ova also the iron is united with a proteid fixed in the yolk spherules, and as this proteid has all the characters of a nuclein, it is, therefore, in all probability, the same compound as that described by Bunge. This nuclein is, apparently, none other than the chromatin diffused from the nucleus of the maturing ovum. If so, it is possible that the original unmodified nuclear chromatin of the ovum contains iron. Again, the fact, that the lower Vertebrata receive the iron which is present in them during larval life in combination with a nuclein, points to the occurrence during the embryonic period in Mammalia of the same or a similar endowment with an iron-holding nuclein. There is, in the Cat, as stated already, a transference of chromatin from the maternal to the fœtal tissues. Judging from all the aspects of these questions it was inferred also that, in the transference of chromatin to the embryo, the latter receives all the iron which it requires.

These points, taken in connexion with the fact that Zaleski isolated from the liver cells of various animals a nuclein in which iron is firmly bound, led me to the generalisation that the chromatin of every cell contains iron as a necessary constituent of itself. I determined to test the accuracy of this generalisation. Under my directions, Mr. R. R. Bensley isolated, from lamb's testicles and the calf's thymus, quantities of nuclein, which, when carefully purified, gave not the slightest iron reaction, but in the ash of which there was abundant evidence of the presence of the metal.*

Although care was taken to obtain the nuclein free from hæmatin, yet I was not certain that we did obtain it absolutely in a pure condition. It is well known that fixed or dead chromatin readily absorbs dyes, and, as it is extremely probable that this power is exercised on other substances, it may well be believed that

* In the preparation of nuclein from these sources we proceeded as follows:—The organs, freed from connective tissue structures, were finely minced and rubbed up in a glass mortar with a small quantity of a 2 per cent. solution of hydrochloric acid. To this fluid, after having been strained through moderately fine muslin, to remove small portions of fibrous tissue, more of the weak solution of the acid was added, with a small quantity of a glycerine extract of the mucosa of the cardiac portion of the pig's stomach. The fluid so prepared was kept at a temperature of 35° C. To the undigested residue left each day fresh quantities of the hydrochloric acid solution and of the glycerine extract were added, until the peptone reaction was no longer obtainable from it. It was next washed with alcohol, then carefully with ether, to remove all the fat, and submitted to the action of strong ammonia for twenty-four hours. A portion was thus dissolved, and the solution, freed from the insoluble part by filtration through iron-free paper, was next treated with three times its volume of alcohol. The precipitate from this was removed, again dissolved in ammonia, and reprecipitated with alcohol. The nuclein so obtained gave no iron to Bunge's fluid, nor did it give to alcohol acidified with sulphuric acid any hæmatin.

hæmatin is as readily absorbed and as tenaciously held by chromatin as are some dyes. Indeed, the accuracy of Zaleski's observations on *hepatin*, the iron-holding nuclein of the liver cells, may be open also to objection on this score. It is, to a certain extent, accepted that the liver is the organ which converts the hæmatin of disintegrated hæmoglobin into the bile and urine pigments. Now, the liver cells are probably the agents in this conversion, and the hæmatin which is held by them and their nuclei cannot, presumably, be removed by the washing out of the lobular capillaries with saline solutions.

Such being the difficulty, the solution appeared, at first sight, to lie in the preparation of nuclein from an animal from which hæmoglobin is absent. No opportunity presented itself for making such preparations of the substance in desired quantities, and it appeared to me that nuclein from such a source could not be free from the histo-hæmatins found by MacMunn to be present in all tissues, except those of the nervous system. With this difficulty before me, I felt compelled to relinquish that line of investigation, and to attempt another.

It occurred to me that it might be possible to demonstrate under the microscope the presence of iron in the chromatin of the cellular elements of such tissues as are almost wholly free from hæmatins, and in those tissues which are hardened so rapidly, as to prevent a transference from without of hæmoglobin or hæmatin into the cells. Such tissues are the cutaneous epithelium in Amphibia and Fishes, the cornea and cartilage in all Vertebrates. This demonstration I had often attempted without success, and I was forced to adopt the conclusion that, if iron is present in the intact nucleus, it is either so firmly bound in the molecules of chromatin that the ordinary reagents cannot attack it, or so small in quantity that its colour reactions are absent, even under the microscope. Bunge, however, in the case of hæmatogen, had shown that ammonium sulphide has the power of separating the iron; and Mr. Bensley and myself ascertained that this reagent has the same effect on isolated chromatin, when kept in a warm condition for a long time in contact with the substance, the iron separating as sulphide. I tried the effect of the reagent on tissues, and took, for example, pieces of the mesentery of *Necturus*, put them in a ground-stoppered bottle with ammonium sulphide, and kept the bottle in a warm oven for several days. At the end of that time, the pieces of tissue showed along the line of the blood-vessels a greenish-blue colour, while the remaining portions had a diffuse light-green colour. Under the microscope, the iron reaction appeared in the red blood cells and their nuclei, while the remaining cellular elements showed nothing further than a diffuse light-green colour in both cell and nucleus. I found, moreover, that the same results were obtained when the preparations were immersed in an

alcoholic solution of ammonium sulphide for twenty-four hours, and kept at the temperature of the laboratory. The pieces of tissue remained for two weeks or more in the warm ammonium sulphide, but no colour reactions appeared in the chromatin of the cells. Not satisfied with the conditions under which the experiments were carried on, I adopted other methods. It occurred to me that, if I kept a few cells for a long time completely separated from one another under a cover-glass, on a slide and surrounded with ammonium sulphide, the reaction might come out. To prevent the evaporation of the ammonium sulphide, I luted the edges of the cover to the slide with various luting compounds, only to find that, in some way or other, the preparation spoiled after a day or two. The luting method being useless, I employed another device. Glycerine, when kept in contact with ammonium sulphide at a moderately high temperature for some time, does not affect the latter reagent, and is itself unaffected. I teased out with clean goose-quill points on a slide a small piece of the testicle of *Necturus*, hardened in 70 per cent. alcohol, added a drop of freshly-prepared ammonium sulphide, put on a cover-glass, allowed a drop of glycerine to run in from its edge, and then placed the slide in a warm oven with a constant temperature of 60° C. Here it was allowed to lie for three days, at the end of which time I examined it under the microscope, and found that, in addition to that reaction distinctly present in the cell body and nucleus of each red corpuscle, there was one apparent in the nuclei of a group of testicular cells at one spot in the preparation. The slide was replaced in the warm oven, and the daily examination of it showed that, accompanying the increase in the number of cells presenting the iron reaction, there was an increase in the depth of the colour in those nuclei first affected, until, at the end of twenty days, the great majority of the testicular nuclei under a cover-glass, 16 mm. square, manifested a colour varying from light green or greenish-blue to dark-green or black. Under a high-power objective the colour was found confined to the chromatin nodules and nuclear network. After three weeks the nuclei adjacent to the edges of the cover-glass began to lose their stained appearance, until, finally, the chromatin possessed only a rusty appearance due to the formation of ferric oxide, for, when a mixture of hydrochloric acid and potassic ferrocyanide was allowed to run under the cover, the rust-coloured nuclei immediately assumed a deep azure-blue colour. That it was the chromatin alone in such cells which presented the reaction with ammonium sulphide was abundantly shown in the karyokinetic figures present in the same preparation. The achromatin and cell substance were unaffected.

Encouraged by the success of this experiment, I made a number of preparations from the other organs of *Necturus*, hardened also in 70 per cent. alcohol. Nearly all of these were successful, but the time

required for the production of the reaction varied greatly. A number of conditions seem to assist in, or retard, and even prevent the success of the experiment; but what are all the favourable conditions I do not know as yet. It is certain, however, that there is a proper proportion of glycerine and ammonium sulphide in the mixture to be added, and I am at present endeavouring to determine what that proportion is. It is also ascertained that the nucleus must be surrounded on all sides by the mixture, otherwise it very rarely shows the reaction. Where the cell body is large, as, for example, in the semi-matured ovarian ova of *Necturus*, the reaction has not yet appeared; while in those very small ova, in which the nucleus, rich in chromatin, forms by far the greater part of the cell, the reaction appears as readily as in the testicular nuclei. Again, the preparation may advance in the reaction up to a certain point, showing the majority of its nuclei possessing a light-green, greenish-blue, or slate colour; when a change occurs, the rust-tint replacing these colours in the chromatin. So far as my experience goes, this happens when too little sulphide has been mixed with the glycerine, or when the sulphide used is beginning to turn deep yellow, or is old.

The deep-yellow sulphide gives no reaction with nuclei, even after eight weeks, while the most active is the freshly prepared reagent. This seems to indicate that the process, by which the iron is set free from the chromatin, is essentially a reducing one, the yellow sulphide having much less reducing power than the colourless, or nearly colourless, reagent. In this way we can explain why the nucleus must be completely surrounded by the reagent, for the reducing capacity of the latter is limited, and a large quantity of it must be concentrated in its action on some particularly small object. When, however, the cellular elements are in a mass, not even the peripherally placed nuclei are affected in the manner described, but they react when they are teased out and separated.

In some tissues there is more or less of albuminate iron or of a deposit of inorganic iron compounds. In such a case the addition of ammonium sulphide gives a reaction immediately. The presence of such compounds sometimes offers a difficulty, especially if they happen to occur in the nuclei. I guarded against confusion in all such cases by submitting thin sections of such tissues made with the free hand to the action of a large quantity of Bunge's fluid for eight to ten hours.* Such sections also, if made from alcohol-hardened tissues with a clean steel knife, covered with absolute alcohol, are not in the

* This fluid consists of ninety volumes of 96 per cent. alcohol and ten volumes of hydrochloric acid, 25 per cent. strength. It, according to Bunge, extracts all inorganic iron compounds and albuminate iron from egg-yolk, and I have found that it removes all traces of such from sections of the spleen, liver, and kidney, which react immediately on the application of acid ferrocyanide solutions.

slightest degree impregnated with iron from the instrument. Zaleski,* has also found that, in sections of the liver made in the usual way, there is nothing in the distribution and quantity of iron present, different from what is observed in sections of the same piece of tissue prepared with a glass knife.

Having ascertained the conditions, generally, under which the presence of iron is successfully demonstrated in chromatin, I tried once more the experiments with those tissues which can readily be obtained free, at least, from hæmoglobin. I found that the reaction came out definitely and distinctly with the chromatin of the corneal and cutaneous epithelium, and in the nuclei of the cartilage cells of *Necturus*. Here, as in the other instances, the time required for the production of the reaction was found to vary greatly, and two preparations from the same organ, *e.g.*, the cornea, presented differences in this respect.

I have succeeded in obtaining the iron reaction in the chromatin of the cells of the following organs and tissues of *Necturus*:—testicle, ovary, gastric and intestinal epithelium, gastric and intestinal glands, pancreas, liver, kidney, cartilage of tongue and shoulder girdle, the cutaneous epithelium, the mesenteric endothelium and connective tissue, and the muscularis of the intestine. I found also the iron reaction in the chromatin of red, white, and fusiform cells of the blood.

From two human placentæ of about five and seven weeks respectively, thoroughly freed from hæmoglobin before hardening, and having the appearance of bleached linen, I removed portions, which I washed carefully with a mixture of alcohol and sulphuric acid to deprive them of traces of hæmatin, and subjected them to the action of warm ammonium sulphide on the slide. The nuclei of all the isolated epithelial cells of such gave a beautiful and intense iron reaction after ten days. Indeed, certain parts of the preparations reminded one of the iodine-green nuclear stain, but after being three weeks in the warm oven, the colour became greenish-black. The nuclei of the hæmatoblasts in the villi give the iron reaction in twenty-four hours, and about two to four days are required to show that the masses situated in the eosinophilous amœbocytes scattered through the connective tissue of the villi also contain iron.

The iron reaction was obtained at the end of two weeks in the nuclei of the epidermal cells of a fœtal kitten removed at about half term from the recently killed cat in a way to prevent the absorption of, or contamination with, hæmoglobin.

Treatment of sections of the placenta of the cat with warm ammonium sulphide also shows, as I expected, that with the passage of chromatin from the maternal to the fœtal tissues, there is also a

* *Loc. cit.*, p. 483.

transference of iron to the embryo. At the base of the placental mucosa, there are glands whose cellular elements pass through a history not unlike in some respects that of the mammary gland. They proliferate, enlarge in size, apparently extrude particles like fat into the lumen of the gland, and then they undergo chromatolysis. The masses of chromatin set free can be readily recognised in the *débris*. In some cases the upper wall of the gland is broken through by the extremity of a villus, whose elongated epithelial cells now stretch amœba-like towards the *débris*, particles of which they invaginate, and among these, chromatin granules. The latter finally reach the centre of the villus, and, with the chromatin obtained from disintegrated maternal endothelial cells, form there a more or less compact column of chromatin. When the embryo measures 25 mm. in length, the amount of chromatin is small, but in considerably later stages it is so abundant that, in stained sections of 30 μ in thickness, the masses formed of it can be seen with the naked eye. In the younger placentæ the chromatin gives, with warm ammonium sulphide, the reaction at the end of twenty-four hours, but the presence of iron is not indicated by hydrochloric acid and potassic ferrocyanide. In the older placentæ, the acid and ferrocyanide mixture gives the iron reaction with the chromatin masses at once, as does also the ammonium sulphide. Now, the chromatin granules in the *débris* of the glands at the base of the placentæ do not in any case give a reaction with the acid mixture, while with warm ammonium sulphide they show the presence of iron after two days. From this it is to be concluded that the chromatin of disintegrated cells manifests more and more readily as time goes on the reaction with ammonium sulphide, and my experiments with this reagent on degenerating cells in other organs confirm this conclusion.

Now in such sections of the placentæ, the chromatin of none of the ordinary cells gives the reaction, even if the sections are kept for weeks in contact with warm ammonium sulphide, either in a bottle or under a covered glass. If the cells are teased out from one another, so that they lie free and separate under the cover, the reaction is obtained in each in about ten days, and it is as distinct as in the chromatin granules in the glandular *débris*, or as in the masses in the central parts of the villi.

I have also obtained the iron reaction in the chromatin in the intestinal cells and the maturing ova of *Oniscus*, in the nuclei of the maturing ova, and of the spermatozoids of *Ascaris mystax*, and in the smaller cells of the larvæ of a species of *Chironomus* found on the stones in running water in the neighbourhood of Toronto in winter. The cells of the salivary gland of the latter animal are too large to give the reaction readily, and, as I write, it has only now begun to appear in the nucleolus in which the chromatin filament terminates,

In the smaller cells, the reaction seems to differentiate between the chromatin and linin parts of the filament.

I may state also, that by this method the iron-holding compound of the muscle-fibre in *Oniscus* is found to be confined to its dim bands.

I have repeatedly employed the hydrochloric acid and potassic ferricyanide mixture to show that the green or greenish-black compound resulting in the nuclei from the action of ammonium sulphide is ferrous sulphide. For this purpose, the nuclei, which are rich in chromatin, *e.g.*, those of the testicular cells and of the immature ovarian ova in *Necturus*, are the best. Usually I washed out the glycerine and sulphide mixture from under the cover-glass by the addition of a large drop of a mixture of equal parts of glycerine and water, and, after some hours, when it had run under the cover, a strip of paper touching the opposite side drained away a portion of this. A repetition of this process several times left very little sulphide, and, very often, few cells under the cover. The addition now of a drop of a mixture of weak hydrochloric acid, and of freshly prepared potassic ferricyanide led to the formation in the previously green or greenish-black nuclei of a deep azure-blue colour, strictly limited to the parts originally affected with the ammonium sulphide. This reaction is sharp, and comes out almost immediately, whereas when hydrochloric acid and potassic ferrocyanide are used, the blue reaction comes out in about half-an-hour, and the colour seems to diffuse through the nucleus and sometimes into the cell. The acid and ferricyanide mixture I have also employed successfully on the nuclei of the cutaneous epithelium, and of the hepatic, gastric, intestinal, and pancreatic cells of *Necturus*, which had previously reacted with ammonium sulphide. In no case was it found that the immediate application of the acid reagent mentioned, or of acid ferrocyanide solution, gave the slightest reaction with those species of nuclei which required a more or less lengthy contact with ammonium sulphide in order to develop the iron reaction. In every particular instance referred to, the latter reagent had to be employed to decompose the chromatin, and set free its iron as sulphide, and the acid mixtures then, and then only, gave a deep azure-blue colour.

Now it might be urged that this iron reaction is due to hæmatin or an allied iron compound. The observations which I now proceed to detail will, I think, completely meet this objection.

Believing that if iron enters into the composition of the chromatin of the animal cell, it must be also present in that of the vegetable cell, I asked Mr. Bensley to employ my method in studying the distribution of iron in the latter. His investigations, so far as they have gone, have confirmed mine, since he has found that the chromatin of the pollen cells of *Dianthus*, *Cucurbita*, *Narcissus*, and of the

cells of pollen sacs of *Hyacinthus*, all fixed in alcohol, give, with several days' application of warm ammonium sulphide, under the cover-glass, a greenish-blue or a dark green reaction.

We have observed that the chromatin of the karyokinetic figures in the pollen grains of *Cucurbita* shows an intense coloration with ammonium sulphide. It has, moreover, been found that there is here, as shown by the application of staining reagents (Ehrlich's hæmatoxylin and Czokor's alum cochineal), a diffusion of the chromatin from one of the two nuclei of the maturing pollen grain into the pollen cell, and this diffusion continues till, finally, there is, comparatively, little chromatin left in the shrunken nucleus.* While the diffusion is taking place, the chromatin is more abundant in the immediate neighbourhood of the nucleus. Now in such maturing pollen grains, hardened in alcohol, there is produced by ammonium sulphide after several days' stay in the warm oven, an iron reaction, corresponding in intensity and distribution with the colour produced by the staining reagents, diffuse in the nucleus, strongly marked in its neighbourhood, and slightly at the periphery of the cell. As the maturation of the pollen grain progresses, the iron reaction is more readily obtainable, and, when the maturation is apparently complete, the pollen cell gives, with freshly prepared ammonium sulphide, in a few hours a light green reaction which becomes but a shade deeper after several days' stay in the warm oven.

Mr. Bensley has also been able to determine with the ferrocyanide mixture the passage of iron salt along the bast portion of the fibro-vascular bundles in the ovary after the opening of the flower, and he has traced these iron salts in sections of the ovary through the raphe of the ovules up to the boundary line of the latter. Beyond this point the iron salts, if they advance, become hidden or disposed of in such a way that they no longer give reactions with acid solutions of potassic ferrocyanide or ferricyanide. Nor do sections of the ovules show any reaction with warm ammonium sulphide, either under a cover-glass or in the bottle. Taught by the experiments on animal cells, I teased out with goose-quill points sections of the ovules in ammonium sulphide and glycerine on the slide, so far as to isolate the various parts of the ovule, and, after keeping the preparation in the warm oven for three days, the nuclei of nearly all the separate and individual cells showed a dark green reaction, which was due to the presence of iron, as the application of a mixture of dilute hydrochloric acid and potassic ferricyanide proved. I have been able in this way to deter-

* A similar diffusion of nuclear substance into the pollen cell takes place, according to Strasburger, in those Angiosperms in which each pollen grain develops numerous pollen tubes ('Sitzungsber. der Niederrhein. Gesell. für Natur- und Heilkunde,' December, 1882, referred to in Just's 'Botanische Jahresberichte' for that year).

mine the presence of iron in the chromatin of a large number of vegetable cells.

I asked Mr. J. J. MacKenzie to undertake the study of the distribution of iron in fungi and algæ, and very encouraging have been, so far, the results of his examination. He has found, for example, that in the gonidia of *Cystopus candidus*, hardened in alcohol, the application of warm ammonium sulphide and glycerine on the slide for eight days brings out the presence of four or more blue-green, round bodies, measuring 1·6 μ in diameter, and corresponding to the nuclei of the zoogonidia, the rest of the protoplasm of the gonidia remaining absolutely uncoloured. The coloured parts gave one the impression as if the gonidia had been given a purely nuclear stain with iodine-green. Mr. MacKenzie has also observed interesting results following the employment of ammonium sulphide on some blue-green algæ, which indicate that here also there is a substance like chromatin in firm combination with iron.

I think that enough has been advanced to show that my view, that the chromatin of every cell, animal and vegetable, is an iron-holding compound, is one which is now capable of proof. I cannot assert that it is proved as yet, since I am aware that that can only be done after an extensive series of observations made and careful work performed. Mr. MacKenzie, Mr. Bensley, and myself are continuing the investigations outlined, and we hope before long to be in a position to bring forward an abundance of interesting facts, which are now accumulating, and of which those given here are examples.

I forego any speculation as to the bearings which my observations may have on our knowledge of animal and vegetable metabolism. I content myself here with referring briefly to the condition in which the iron is present in the chromatin. As stated already, I have found that the hæmoglobin of Amphibia is formed from the chromatin of the hæmatoblasts. This would seem to indicate that the iron is attached in the chromatin molecule to an atom-group somewhat like that of hæmatin. As the oxygen-carrying property of hæmoglobin is generally attributed to the presence of the iron in it, we may ask ourselves whether the chemical processes in the chromatin of the living cell are due to a constant alternation of the oxidised and reduced conditions of the iron in the chromatin molecule. As hæmoglobin results from degeneration or disintegration of chromatin, we would naturally expect to find in it one or other condition specially prominent. The more stable condition is that of oxidation. It is possible that in living chromatin the conditions are more readily interchangeable, and that therein lies a basis for a theory of those chemical processes of the cell which are grouped under the term "vital."